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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/11, 9/00, 15/86, C07H 21/00, A61K 31/70 // 48/00

A2

(11) International Publication Number: **WO 97/10334**

(43) International Publication Date: 20 March 1997 (20.03.97)

(21) International Application Number: PCT/US96/14838

(22) International Filing Date: 12 September 1996 (12.09.96)

(30) Priority Data:
08/527,060 12 September 1995 (12.09.95) US

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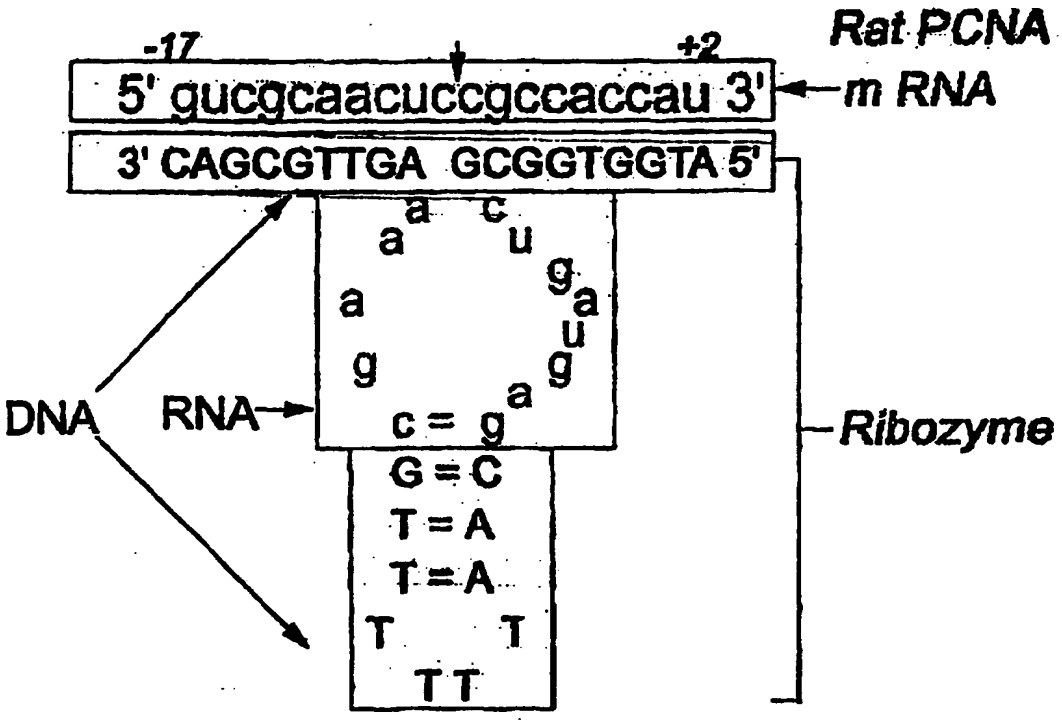
(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: RIBOZYME THERAPY FOR THE TREATMENT AND/OR PREVENTION OF RESTENOSIS



(57) Abstract

As an effective therapy for restenosis, this invention provides ribozymes and ribozyme delivery systems useful to inhibit abnormal smooth muscle cell proliferation in vascular tissue. Methods of producing ribozymes and gene therapy utilizing these ribozymes also are provided. The ribozymes are especially targeted to cdc2-kinase PCNA, cyclin B1, lysyl oxidase or an extracellular matrix protein.

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Description

RIBOZYME THERAPY FOR THE TREATMENT AND/OR PREVENTION OF RESTENOSIS

Technical Field

The present invention relates generally to therapeutics, and more specifically, to compositions and methods which may be utilized in the treatment and/or prevention of restenosis.

Background of the Invention

In 1992, an excess of 300,000 angioplasties were performed in the United States. Restenosis is a major complication following angioplasty, occurring in 30%-60% of patients. Indeed, restenosis is the single most significant problem in interventional cardiology and costs the health care system in excess of $1 billion per year.

Restenosis following angioplasty is the result of local vascular injury, and is characterized by the local infiltration of platelets and macrophages, and local activation of the clotting system. These factors result in the elaboration of a number of biologic mediators of smooth muscle cell (SMC) migration and proliferation. These SMCs migrate into the vascular intima and begin to proliferate and produce extracellular matrix (ECM), resulting in the formation of a fibrocellular mass which can obstruct blood flow. Further, injury has been shown to induce the expression of a variety of oncogenes that are believed to play a role in the cellular response to this injury. Thus, a need exists for an effective therapy to prevent and treat restenosis. The present invention satisfies this need and further provides other, related, advantages as well.

Summary of the Invention

As an effective therapy for restenosis, this invention provides ribozymes and ribozyme delivery systems useful to inhibit abnormal smooth muscle cell

proliferation in vascular tissue. Methods of producing ribozymes and gene therapy utilizing these ribozymes also are provided.

Accordingly, in one aspect the present invention ribozymes having the ability to inhibit abnormal smooth muscle cell proliferation in vascular tissue. Preferably, the ribozyme is a hammerhead or hairpin ribozyme, representative examples of which recognize the target site sequences set forth below and in the Examples. In preferred embodiments, the present invention also provides nucleic acid molecule encoding such ribozymes; further preferably, the nucleic acid is DNA or cDNA. Even further preferably, the nucleic acid molecule is under the control of a promoter to transcribe the nucleic acid.

In another aspect, the present invention provides host cells containing the ribozymes described herein, vectors comprising the nucleic acid encoding the ribozymes described herein, and host cells comprising such a vector. Preferably, the vector is a plasmid, retrotransposon, a cosmid or a virus (*e.g.*, adenovirus, adeno-associated virus, or a retrovirus). In one embodiment where the vector is a retroviral vector, the nucleic acid molecule encoding the ribozyme under the control of a promoter, which is preferably a pol III promoter, further preferably a human $tRNA^{Val}$ promoter or an adenovirus VA1 promoter, is inserted between the 5' and 3' long terminal repeat sequences of the retrovirus.

The present invention also provides a host cell stably transformed with such a retroviral vector. Preferably, the host cell is a murine or a human cell.

In a further aspect, the present invention provides methods for producing a ribozyme, the ribozyme being able to inhibit abnormal smooth muscle cell proliferation in vascular tissue, which method comprises providing a nucleic acid molecule (*e.g.*, DNA) encoding the ribozyme under the transcriptional control of a promoter, and transcribing the nucleic acid molecule to produce the ribozyme. Preferably, the method further comprises purifying the ribozyme produced. The ribozyme may be produced *in vitro*, *in vivo* or *ex vivo*.

In yet another aspect, the present invention provides methods of inhibiting abnormal smooth muscle cell proliferation in vascular tissue, which method comprises introducing into the cell an effective amount of the ribozymes described

herein. In one embodiment, such methods comprise introducing into the cell an effective amount of DNA encoding a ribozyme as described herein and transcribing the DNA to produce the ribozyme. Preferably, the cell is a human cell.

In still a further aspect, the present invention provides methods of preventing abnormal smooth muscle cell proliferation in vascular tissue, which methods comprise introducing into the cell an effective amount of a nucleic acid molecule (*e.g.*, DNA) encoding a ribozyme as described herein and transcribing the DNA to produce the ribozyme. Preferably, the cell is a human cell.

In preferred embodiments, the methods further comprise administering the cell transduced with a retroviral vector to a mammal of the same species as that from which the transduced cell was obtained. In other preferred embodiments, the cell transduced with the retroviral vector has been obtained from the mammal receiving the transduced cell.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth herein that describe in more detail certain procedures or compositions (*e.g.*, plasmids, etc.), and are therefore incorporated by reference in their entirety as if each were individually noted for incorporation.

Brief Description of the Drawings

Figure 1 is a schematic illustration of vector pLNT-Rz.

Figure 2 is a schematic illustration of a representative hairpin ribozyme.

Figure 3 is a schematic illustration of an adenoviral vector expressing a hairpin ribozyme gene.

Figure 4 is a graph which illustrates the effects of ribozymes on a balloon injured rat carotid artery.

Figure 5 is a graph which illustrates the effects of ribozymes on a balloon injured rat carotid artery.

Figure 6 is a schematic illustration of a ribozyme directed against rat PCNA.

Figure 7 is a schematic illustration of a ribozyme directed against rat cdc-2 kinase.

Detailed Description of the Invention

DEFINITIONS

Prior to setting forth the invention, it may be helpful to an understanding thereof to first set forth definitions of certain terms that will be used hereinafter.

"Ribozyme" refers to a nucleic acid molecule which is capable of cleaving a specific nucleic acid sequence. Ribozymes may be composed of RNA, DNA, nucleic acid analogues (*e.g.*, phosphorothioates), or any combination of these (*e.g.*, DNA/RNA chimerics). Within particularly preferred embodiments, a ribozyme should be understood to refer to RNA molecules that contain anti-sense sequences for specific recognition, and an RNA-cleaving enzymatic activity.

"Ribozyme gene" refers to a nucleic acid molecule (*e.g.*, DNA) consisting of the ribozyme sequence which, when transcribed into RNA, will yield the ribozyme.

"Vector" refers to an assembly which is capable of expressing a ribozyme of interest. The vector may be composed of either deoxyribonucleic acids ("DNA") or ribonucleic acids ("RNA"). Optionally, the vector may include a polyadenylation sequence, one or more restriction sites, as well as one or more selectable markers such as neomycin phosphotransferase, hygromycin phosphotransferase or puromycin-N-acetyl-transferase. Additionally, depending on the host cell chosen and the vector employed, other genetic elements such as an origin of replication, additional nucleic acid restriction sites, enhancers, sequences conferring inducibility of transcription, and selectable markers, may also be incorporated into the vectors described herein.

Restenosis is a major clinical problem and as the result of a need for repeat hospitalization, repeat angioplasty or bypass surgery, restenosis costs the nation's health care system in excess of $1 billion per year. Restenosis is believed to comprise three important components. First, myointimal proliferation of vascular smooth muscle cells and the subsequent deposition of ECM results in a fibrocellular mass which can encroach upon the vascular lumen. Second, following acute angioplasty, there may be

significant elastic recoil of the artery which contributes to a late loss of luminal dimension. Finally, platelets and thrombus adherent to the vascular wall may, over time, organize into a fibrocellular mass.

Smooth muscle cells (SMCs) are capable of producing and responding to a variety of growth factors including platelet-derived growth factor (PDGF), transforming growth factor (TGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), and interleukins. These same factors have been found in human restenotic lesions. In addition, a variety of oncogenes (*e.g.*, c-*myc*, c-*fos*, and c-*myb*) have been found to be involved in smooth muscle cell migration and proliferation as well as deposition of ECM that is associated with post-vascular injury. Smooth muscle cells themselves are capable of regulating their own growth by local autocrine and paracrine mechanisms.

As discussed in more detail below, by interfering with the local production and action of the growth factors, oncogenes and cell regulatory proteins involved with SMC growth following vascular injury, restenosis can be effectively treated and/or prevented. This invention accomplishes such by providing ribozymes and methods of using ribozymes that directly block the production of the growth factors, oncogenes and cell regulatory proteins involved with SMC growth following vascular injury.

RIBOZYMES

As noted above, the present invention provides ribozymes having the ability to inhibit, prevent or delay the formation of pathologic smooth muscle proliferation and restenosis. Several different types of ribozymes may be constructed for use within the present invention, including for example, hammerhead ribozymes (Rossi, J.J. et al., *Pharmac. Ther. 50*:245-254, 1991) (Forster and Symons, *Cell 48*:211-220, 1987; Haseloff and Gerlach, *Nature 328*:596-600, 1988; Walbot and Bruening, *Nature 334*:196, 1988; Haseloff and Gerlach, *Nature 334*:585, 1988; Haseloff et al., U.S. Patent No. 5,254,678), hairpin ribozymes (Hampel et al., *Nucl. Acids Res. 18*:299-304, 1990, and U.S. Patent No. 5,254,678), hepatitis delta virus ribozymes (Perrotta and Been, *Biochem. 31*:16, 1992), Group I intron ribozymes (Cech et al., U.S. Patent No.

4,987,071) RNase P ribozymes (Takada et al., *Cell 35*:849, 1983); (*see e.g.*, WO 95/29241, entitled "Ribozymes with Product Ejection by Strand Displacement"; and WO 95/31551, entitled "Novel Enzymatic RNA Molecules."

Cech et al. (U.S. Patent No. 4,987,071, issued January 22, 1991) has disclosed the preparation and use of certain synthetic ribozymes which have endoribonuclease activity. These ribozymes are based on the properties of the *Tetrahymena* ribosomal RNA self-splicing reaction and require an eight base pair target site with a requirement for free guanosine or guanosine derivatives. A temperature optimum of 50°C is reported for the endoribonuclease activity. The fragments that arise from cleavage contain 5'-phosphate and 3'-hydroxyl groups and a free guanosine nucleotide added to the 5'-end of the cleaved RNA. In contrast, the ribozymes of this invention hybridize efficiently to target sequences at physiological temperatures, making them suitable for use *in vivo*, not merely as research tools (*see* column 15, lines 18 to 42, of Cech et al., U.S. Patent No. 4,987,071).

Particularly preferred ribozymes for use within the present invention are hairpin ribozymes (for example, as described by Hampel et al., European Patent Publication No. 0 360 257, published March 26, 1990). Briefly, the sequence requirement for the hairpin ribozyme is any RNA sequence consisting of NNNBN*GUC(N)$_x$ (Sequence ID Nos. 1-5) (where x is any number from 6 to 10, N*G is the cleavage site, B is any of G, C, or U, and N is any of G, U, C, or A). Representative examples of recognition or target sequences for hairpin ribozymes are set forth below in the Examples. Additionally, the backbone or common region of the hairpin ribozyme can be designed using the nucleotide sequence of the native hairpin ribozyme (Hampel et al., *Nucl. Acids Res. 18*:299-304, 1990) or it can be modified to include a "tetraloop" structure that increases stability and catalytic activity (see Yu et al., *Virology 206*:381-386, 1995; Cheong et al., *Nature 346*:680-682, 1990; Anderson et al., *Nucl. Acids Res. 22*:1096-1100, 1994). The sequence requirement at the cleavage site for the hammerhead ribozyme is any RNA sequence consisting of NUX (where N is any of G, U, C, or A and X represents C, U, or A) can be targeted. Accordingly, the same target within the hairpin leader sequence, GUC, is useful for the hammerhead ribozyme. The additional nucleotides of the hammerhead ribozyme or hairpin ribozyme is

determined by the target flanking nucleotides and the hammerhead consensus sequence (*see* Ruffner et al., *Biochemistry 29*:10695-10702, 1990).

The above information, along with the sequences and disclosure provided herein, enables the production of hairpin ribozymes of this invention. Appropriate base changes in the ribozyme are made to maintain the necessary base pairing with the target sequences. In one embodiment, the ribozymes provided herein have the ability to inhibit the activity of a growth factor responsible for abnormal smooth muscle cell proliferation in vascular tissue. Such growth factors include, but are not limited to platelet derived growth factor, fibroblast growth factor, insulin-like growth factor, c-*myc*, c-*myb*, c-*fos*, cdc2 kinase (Genbank accession number Y00272; Lee and Nurse, *Nature 327*:31–35, 1987), proliferating cell nuclear antigen ("PCNA" Genbank accession number J04718; Travali et al., *G. Biol. Chem. 264*(13):7466-7472, 1989) , Cyclin B1 (Genbank accession number M25753; Pines and Hunter, *Cell 58*:833–846, 1989), Lysyl Oxidase (Genbank accession number M94054), TGF-α and TGF-β proteins, interleukins, and components of the extracellular matrix. As used herein, the term "abnormal smooth muscle cell proliferation" should be understood to mean any small cell proliferation and deposition of extracellular matrix occurring in response to vascular injury or trauma involved by any angioplasty, stent, balloon angioplasty, atherectomy, laser surgery, endovascular or surgical procedure.

The ribozymes of this invention, as well as DNA encoding such ribozymes and other suitable nucleic acid molecules, described in more detail below, can be chemically synthesized using methods well known in the art for the synthesis of nucleic acid molecules (*see e.g.*, Heidenreich et al., *J. FASEB 70*(1):90-6, 1993; Sproat, *Curr. Opin. Biotechnol. 4*(1):20-28, 1993). Alternatively, Promega, Madison, Wis., USA, provides a series of protocols suitable for the production of nucleic acid molecules such as ribozymes.

Within other aspects of the present invention, ribozymes can also be prepared from a DNA molecule or other nucleic acid molecule (which, upon transcription, yields an RNA molecule) operably linked to an RNA polymerase promoter, *e.g.*, the promoter for T7 RNA polymerase or SP6 RNA polymerase. Accordingly, also provided by this invention are nucleic acid molecules, *e.g.*, DNA or

cDNA, coding for the ribozymes of this invention. When the vector also contains an RNA polymerase promoter operably linked to the DNA molecule, the ribozyme can be produced *in vitro* upon incubation with the RNA polymerase and appropriate nucleotides. In a separate embodiment, the DNA may be inserted into an expression cassette, such as described in Cotten and Birnstiel, *EMBO J.* 8(12):3861-3866, 1989, and in Hempel et al., *Biochemistry* 28:4929-4933, 1989. A more detailed discussion of molecular biology methodology is disclosed in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, 1989.

During synthesis, the ribozyme can be modified by ligation to a DNA molecule having the ability to stabilize the ribozyme and make it resistant to RNase (Rossi et al., *Pharmac. Ther.* 50:245-254, 1991). Alternatively, the ribozyme can be modified to the phosphothio analog for use in liposome delivery systems. This modification also renders the ribozyme resistant to endonuclease activity.

VECTORS

Use of ribozymes to treat abnormal smooth muscle cell proliferation involves introduction of functional ribozyme to the cells of interest. This can be accomplished by either synthesizing functional ribozyme *in vitro* prior to delivery, or, by delivery of DNA capable of driving ribozyme synthesis *in vivo*.

More specifically, within other aspects of the invention the ribozyme gene may be constructed within a vector which is suitable for introduction to a host cell (*e.g.*, prokaryotic or eukaryotic cells in culture or in the cells of an organism). Appropriate prokaryotic and eukaryotic cells can be transfected with an appropriate transfer vector containing the nucleic acid molecule encoding a ribozyme of this invention.

To produce the ribozymes with a vector *in vivo*, nucleotide sequences coding for ribozymes are preferably placed under the control of a eukaryotic promoter such as pol III (*e.g.*, tRNA), CMV, SV40 late, or SV40 early promoters. Within certain embodiments, the promoter may be a tissue-specific promoter such as, for example, the albumin promoter and the alphafetoprotein promoter (Feuerman et al., *Mol. Cell. Biol.* 9:4204-12, 1989; Camper and Tilghman, *Genes Develop.* 3:537-46, 1989); the alcohol

dehydrogenase promoter (Felder, *Proc. Natl. Acad. Sci. USA 86*:5903-07, 1989); the Apolipoprotein B gene promoter (Das et al., *J. Biol. Chem. 263*:11452-8, 1988); the Coagulation protease factor VII gene promoter (Erdmann et al., *J. Biol. Chem. 270*:22988-96, 1995); the Fibrinogen gamma gene promoter (Zhang et al., *J. Biol. Chem. 270*:24287-91, 1995); the Glucokinase gene promoter (Williams et al., *Biochem. Biophys., Res. Comm. 212*:272-9, 1995); the Liver phosphofructokinase gene promoter (Levanon et al., *Biochem. Mol. Biol. Int.* 35:729-36, 1995); the Phospho-Enol-Pyruvate Carboxy-Kinase ("PEPCK") promoter (Hatzogiou et al., *J. Biol. Chem. 263:* 17798-808, 1988; Benvenisty et al., *Proc. Natl. Acad. Sci. USA 86*:1118-22, 1989; Vaulont et al., *Mol. Cell. Biol. 9*:4409-15, 1989); or lymphoid-specific promoters. Ribozymes may thus be produced directly from the transfer vector *in vivo*.

A wide variety of vectors may be utilized within the context of the present invention, including for example, plasmids, viruses, retrotransposons and cosmids. Representative examples include adenoviral vectors (*e.g.*, WO 94/26914, WO 93/9191; Yei et al., *Gene Therapy 1*:192-200, 1994; Kolls et al., *PNAS 91*(1):215-219, 1994; Kass-Eisler et al., *PNAS 90*(24):11498-502, 1993; Guzman et al., *Circulation 88*(6):2838-48, 1993; Guzman et al., *Cir. Res. 73*(6):1202-1207, 1993; Zabner et al., *Cell 75*(2):207-216, 1993; Li et al., *Hum Gene Ther. 4*(4):403-409, 1993; Caillaud et al., *Eur. J. Neurosci. 5*(10):1287-1291, 1993), adeno-associated type 1 ("AAV-1") or adeno-associated type 2 ("AAV-2") vectors (*see* WO 95/13365; Flotte et al., *PNAS 90*(22):10613-10617, 1993), hepatitis delta vectors, live, attenuated delta viruses and herpes viral vectors (*e.g.*, U.S. Patent No. 5,288,641), as well as vectors which are disclosed within U.S. Patent No. 5,166,320. Other representative vectors include retroviral vectors (*e.g.*, EP 0 415 731; WO 90/07936; WO 91/02805; WO 94/03622; WO 93/25698; WO 93/25234; U.S. Patent No. 5,219,740; WO 93/11230; WO 93/10218). Methods of using such vectors in gene therapy are well known in the art, see, for example, Larrick, J.W. and Burck, K.L., *Gene Therapy: Application of Molecular Biology*, Elsevier Science Publishing Co., Inc., New York, New York, 1991 and Kreigler, M., *Gene Transfer and Expression: A Laboratory Manual*, W.H. Freeman and Company, New York, 1990.

Further provided by this invention are vectors having more than one nucleic acid molecule encoding a ribozyme of this invention, each molecule under the control of a separate eukaryotic promoter or alternatively, under the control of single eukaryotic promoter. Representative examples of other therapeutic molecules which may be delivered by the vectors of the present invention include interferon (*e.g.*, alpha, beta or gamma), as well as a wide variety of other cytokines or growth factors. These vectors provide the advantage of providing multi-functional therapy against abnormal smooth muscle cell proliferation, preferably with the various therapies working together in synergy.

Host cells containing the ribozymes, nucleic acids and/or vectors described above also are within the scope of this invention. These host cells can be procaryotic cells, for example bacterial cells or eucaryotic cells, such as mammalian, human, rat, or mouse cells. The host cells transduced with nucleic acids encoding the ribozymes are useful to recombinantly produce the ribozymes. Thus, also provided by this invention is a method for producing a ribozyme, *in vitro* or *in vivo*, the ribozyme being able to inhibit abnormal smooth muscle cell proliferation in vascular tissue. DNA encoding the ribozyme is provided to the cell, the DNA being under the transcriptional control of a promoter, using methods well known to those of skill in the art. See Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory (1989), incorporated herein by reference. The DNA is then transcribed in the cell to produce the ribozyme. When produced *in vitro*, the ribozyme can be purified or isolated from the cell by using methods well known in the art.

DELIVERY

Within certain aspects of the invention, ribozyme molecules, or nucleic acid molecules which encode the ribozyme, may be introduced into a host cell utilizing a vehicle, or by various physical methods. Representative examples of such methods include transformation using calcium phosphate precipitation (Dubensky et al., *PNAS 81*:7529-7533, 1984), direct microinjection of such nucleic acid molecules into intact target cells (Acsadi et al., *Nature 352*:815-818, 1991), and electroporation whereby cells suspended in a conducting solution are subjected to an intense electric field in order to

transiently polarize the membrane, allowing entry of the nucleic acid molecules. Other procedures include the use of nucleic acid molecules linked to an inactive adenovirus (Cotton et al., *PNAS 89*:6094, 1990), lipofection (Felgner et al., *Proc. Natl. Acad. Sci. USA 84*:7413-7417, 1989), microprojectile bombardment (Williams et al., *PNAS 88*:2726-2730, 1991), polycation compounds such as polylysine, receptor specific ligands, liposomes entrapping the nucleic acid molecules, spheroplast fusion whereby *E. coli* containing the nucleic acid molecules are stripped of their outer cell walls and fused to animal cells using polyethylene glycol, viral transduction, (Cline et al., *Pharmac. Ther. 29*:69, 1985; and Friedmann et al., *Science 244*:1275, 1989), and DNA ligand (Wu et al, *J. of Biol. Chem. 264*:16985-16987, 1989). In one embodiment, the ribozyme is introduced into the host cell using a liposome.

PHARMACEUTICAL COMPOSITIONS

As noted above, pharmaceutical compositions also are provided by this invention. These compositions contain any of the above described ribozymes, DNA molecules, vectors or host cells, along with a pharmaceutically or physiologically acceptable carrier, excipients or diluents. Generally, such carriers should be nontoxic to recipients at the dosages and concentrations employed. Ordinarily, the preparation of such compositions entails combining the therapeutic agent with buffers, antioxidants such as ascorbic acid, low molecular weight (less than about 10 residues) polypeptides, proteins, amino acids, carbohydrates including glucose, sucrose or dextrins, chelating agents such as EDTA, glutathione and other stabilizers and excipients. Neutral buffered saline or saline mixed with nonspecific serum albumin are exemplary appropriate diluents.

In addition, the pharmaceutical compositions of the present invention may be prepared for administration by a variety of different routes, including for example intraarticularly, intracranially, intradermally, intrahepatically, intramuscularly, intraocularly, intraperitoneally, intrathecally, intravenously (*e.g.*, into the portal vein), or subcutaneously. In addition, pharmaceutical compositions of the present invention may be placed within containers, along with packaging material which provides instructions regarding the use of such pharmaceutical compositions. Generally, such instructions will

include a tangible expression describing the reagent concentration, as well as within certain embodiments, relative amounts of excipient ingredients or diluents (*e.g.*, water, saline or PBS) which may be necessary to reconstitute the pharmaceutical composition

THERAPEUTIC METHODS

Methods of interfering with, preventing, or inhibiting abnormal smooth cell vascular tissue proliferation or restenosis are also provided by this invention. Such methods require contacting the cell with an effective amount of ribozyme of this invention or, alternatively, by transducing the cell with an effective amount of vector having a nucleic acid molecule encoding the ribozyme. Effective amounts are easily determined by those of skill in the art using well known methodology. When exogenously delivering the ribozyme, the RNA molecule can be embedded within a stable RNA molecule or in another form of protective environment, such as a liposome. Alternatively, the RNA can be embedded within RNase-resistant DNA counterparts. Cellular uptake of the exogenous ribozyme can be enhanced by attaching chemical groups to the DNA ends, such as cholesteryl moieties (Letsinger et al., *P.N.A.S., U.S.A.*, 1989).

In another aspect of the invention, the target cell is transduced under conditions favoring insertion of the vector into the target cell and stable expression of the nucleic acid encoding the ribozyme. The target cell can include but is not limited to vascular smooth muscle cells or cells responsible for the deposition of proteins involved in the formation of extracellular matrix.

Accordingly, another aspect of this invention provides methods for interfering with or preventing abnormal smooth muscle proliferation in a suitable cell, by reacting the target RNA sequence with a ribozyme of this invention. Within the cell or within the cells of an organism, a transfer vector as described above encoding one or more ribozymes is transfected into a cell or cells using methods described in Llewwllyn et al., (1987) *J. Mol. Biol. 195*:115-123 and Hanahan et al. (1983) 166:557-580, each incorporated herein by reference. Inside the cell, the transfer vector replicates and the DNA coding for the ribozyme is transcribed by cellular polymerases to produce ribozymes which then inactivate factors responsible for abnormal smooth muscle cell

proliferation, abnormal extracellular matrix deposition and restenosis. Micromanipulation techniques such as microinjection also can be used to insert the vector into the cell so that the transfer vector or a part thereof is integrated into the genome of the cell. Transcription of the integrated material gives rise to ribozymes which then inactivate the target proteins. As used herein, the term "inactivate" is intended to mean interfere with the production of the protein products such as those discussed above (*e.g.*, c-*myc* or TGF-β).

An alternative method of inhibiting abnormal smooth muscle cell proliferation in vascular tissue consists of introducing into a cell an effective amount of DNA encoding a ribozyme described above, under conditions favoring transcription of the DNA to produce the ribozyme. This method also is useful to prevent abnormal SMC and abnormal extracellular matrix deposition and thus prevent restenosis. The DNA can be transferred in a carrier or in a vector in a carrier a number of ways. For example, the DNA can be administered by transluminal delivery to the vascular wall, or exoluminally. In another aspect, the active DNA can be embedded in a biodegradable polymer or sphere and administered by vascular stent. Alternatively, it can be delivered in a pleuronic gel.

Also provided by this invention is a method of inhibiting or preventing abnormal smooth muscle cell proliferation in vascular tissue in a subject (*e.g.*, a warm-blooded animal such as a human) comprising the step of administering to the subject an effective amount of a ribozyme having the ability to inhibit abnormal smooth muscle cell proliferation in vascular tissue. The ribozyme is delivered to the smooth muscle cell exoluminally, transluminally, by stent, by a biodegradable polymer or sphere or in a pleuronic gel.

The following examples are offered by way of illustration, and not by way of limitation.

EXAMPLES

EXAMPLE 1

CRITERIA FOR RIBOZYME SITE SELECTION

A. Selection of Sites for Hairpin Ribozymes

Within certain embodiments of the invention, hairpin ribozymes suitable for use within the present invention are provided which recognize the following sequence of RNA: NNNBNGUCNNNNNNNN (Sequence I.D. No. 3) wherein the ribozyme is constructed so as to be complementary to the underlined sequences, and wherein B is C, G or U. The sequence GUC must be conserved for all hairpin ribozymes described below. Other nucleotides ("N" as underlined above) preferably have a high degree of sequence conservation in order to limit the need for multiple ribozymes against the same target site. Representative GUC hairpin ribozyme recognition sites for various genes are provided below in Tables 1-4.

Table 1
Hairpin Ribozyme Recognition Sites for cdc 2 kinase

NUCL. POS.	SEQUENCE (5' to 3')	I.D. No.
193	AGTCAGTCTTCAGGAT	6
289	TCCTGGTCAGTACATG	7
530	CTGGGGTCAGCTCGTT	8

Table 2
Hairpin Ribozyme Recognition Sites for Cyclin B1

NUCL. POS.	SEQUENCE (5' to 3')	I.D. No.
12	TCCGAGTCACCAGGAA	9
281	CCAGTGTCTGAGCCAG	10
427	CCTGTGTCAGGCTTTC	11
558	AAGCAGTCAGACCAAA	12
580	ACTGGGTCGGGAAGTC	13
678	TGACTGTCTCCATTAT	14

Table 3
Hairpin Ribozyme Recognition Sites for PCNA

NUCL. POS.	SEQUENCE (5' to 3')	I.D. No.
18	GCCTGGTCCAGGGCTC	15
125	GACTCGTCCCACGTCT	16
158	CTGCGGTCTGAGGGCT	17
867	TTTCTGTCACCAAATT	18

Table 4
Hairpin Ribozyme Recognition Sites for Lysyl Oxidase

NUCL. POS.	SEQUENCE (5' to 3')	I.D. No.
225	CCGCCGTCCCTGGTGC	19
333	CTGGAGTCACCGCTGG	20
364	CGCCCGTCACTGGTTC	21
631	GTACGGTCTCCCAGAC	22
671	CAGGCGTCCACGTACG	23
730	AAACTGTCTGGCCAGT	24
970	TTTCTGTCTTGAAGAC	25

B. Selection of Cleavage Sites for Hammerhead Ribozymes

Hammerhead ribozymes suitable for use within the present invention preferably recognize the sequence NUX, wherein N is any of G, U, C, or A and X is C, U, or A. Representative hammerhead target sites include:

Table 5
Hammerhead Ribozyme Recognition Sites for cdc 2 kinase

NUCL. POS.	SEQUENCE (5' to 3')	I.D. No.
81	TACAGGTCAAGTGGTA	26
159	AAATTTCTCTATTAAAG	27
195	AGTCAGTCTTCAGGAT	6
532	CTGGGGTCAGCTCGTT	8

Table 6
Hammerhead Ribozyme Recognition Sites for Cyclin B1

NUCL. POS.	SEQUENCE (5' to 3')	I.D. No.
14	TCCGAGTCACCAGGAA	9
283	CCAGTGTCTGAGCCAG	10
429	CCTGTGTCAGGCTTTC	11
560	AAGCAGTCAGACCAAA	12
582	ACTGGGTCGGGAAGTC	13
680	TGACTGTCTCCATTAT	14

Table 7
Hammerhead Ribozyme Recognition Sites for PCNA

NUCL. POS.	SEQUENCE (5' to 3')	I.D. No.
20	GCCTGGTCCAGGGCTC	15
127	GACTCGTCCCACGTCT	16
160	CTGCGGTCTGAGGGCT	17
869	TTTCTGTCACCAAATT	18

Table 8
Hammerhead Ribozyme Recognition Sites for Lysyl Oxidase

NUCL. POS.	SEQUENCE (5' to 3')	I.D. No.
227	CCGCCGTCCCTGGTGC	19
335	CTGGAGTCACCGCTGG	20
366	CGCCCGTCACTGGTTC	21
633	GTACGGTCTCCCAGAC	22
673	CAGGCGTCCACGTACG	23
732	AAACTGTCTGGCCAGT	24
972	TTTCTGTCTTGAAGAC	25

EXAMPLE 2

CONSTRUCTION OF HAIRPIN RIBOZYMES

Two single-stranded DNA oligonucleotides are chemically synthesized such that, when combined and converted into double-stranded DNA, they contain the

entire hairpin ribozyme, including nucleotides complementary to the target site. In addition, restriction enzyme recognition sites may be placed on either end to facilitate subsequent cloning. More specifically, the oligonucleotides are hybridized together and converted to double-stranded DNA using either Klenow DNA polymerase or Taq DNA polymerase. The resulting DNA is cleaved with restriction enzymes *Bam*HI and *Mlu*I, purified and cloned into vectors for *in vitro* transcription (pGEM, ProMega, Madison, Wis.) or for retrovirus production and mammalian expression (pLNL/MJT backbone). Representative hairpin ribozymes are set forth below (note that the underlined sequences indicate the sites wherein the ribozyme binds the target sequence):

cdc-2 530 (Sequence I.D. No. 28)

5' AACGAGCTAGAACCAGACCAGAGAAACACACGTTGTGGTATATTACCTGGTA 3'

Cyclin B1 281 (Sequence I.D. No. 29)

5' CTGGCTCAAGAACTGGACCAGAGAAACACACGTTGTGGTATATTACCTGGTA 3'

Lysyl Oxidase 333 (Sequence I.D. No. 30)

5' CCAGCGGTAGAACCAGACCAGAGAAACACACGTTGTGGTATATTACCTGGTA 3'

PCNA 158 (Sequence I.D. No. 31)

5' AGCCCTCAAGAAGCAGACCAGAGAAACACACGTTGTGGTATATTACCTGGTA 3'

Defective ribozymes for use as controls may be constructed as described above, with the exception that the sequence AAA is changed to a UGC as shown in Figure 2.

EXAMPLE 3

CONSTRUCTION OF HAMMERHEAD RIBOZYMES

Chimeric hammerhead ribozymes (*i.e.*, RNA/DNA hybrids) are designed to have an appropriate NUX sequence for ribozyme cleavage. Briefly, ribozymes are chemically synthesized for target sequences of the rat CDC2 kinase gene (nucleotide sequence from -30 to -14), rat PCNA (nucleotide sequence from -17 to +2). (See Figures 6 and 7.) In addition, the following human hammerhead ribozymes are synthesized.

cdc-2 532 (Sequence I.D. No. 32)

5' AACGAGCTCTGATGACCTCGTGAGAGGGAAACCCCAG 3'

Cyclin B1 283 (Sequence I.D. No. 33)

5' CTGGCTCACTGATGACCTCGTGAGAGGGAAACACTGG 3'

Lysyl Oxidase 335 (Sequence I.D. No. 34)

5' CCAGCGGTCTGATGACCTCGTGAGAGGGAAACTCCAG 3'

PCNA 160 (Sequence I.D. No. 35)

5' AGCCCTCACTGATGACCTCGTGAGAGGGAAACCGCAG 3'

A scrambled sequence polynucleotide including the same composition of ribonucleotides and deoxyribonucleotides is also synthesized for each ribozyme to serve as a control with no catalytic activity. Lipofectin may be utilized to enhance the uptake of ribozyme into the cells.

EXAMPLE 4

CONSTRUCTION OF RIBOZYME MAMMALIAN EXPRESSION VECTORS

Plasmid pMJT (Yu et al., *Proc. Nat'l Acad. Sci. USA 90*:6340-6344, 1993), which contains the anti-U5 HIV ribozyme driven by the $tRNA^{val}$ RNA pol III promoter, is digested with *Bam*HI and *Mlu*I, and the vector purified from the ribozyme fragment. The hairpin ribozymes, as described above, are excised from the pGem vector with *Bam*HI and *Mlu*I, purified, and ligated into the empty pMJT vector. The resulting vector is designated pLNT-Rz (*see* Figure 1), and contains the Moloney LTR driving the neomycin resistance gene and the $tRNA^{val}$ RNA pol III promoter driving expression of the ribozyme.

EXAMPLE 5

CONSTRUCTION OF ADENOVIRAL VECTORS

A. Construction of an adenoviral vector expressing ß-galactosidase as a reporter gene (AvCLacZ).

As described in more detail below, a shuttle plasmid pAvCLacZ required for generation of adenoviral vectors (AV). Briefly, pAvCLacZ, a pBR322-based plasmid was constructed that contains: nucleotide base (nt) 1-452 of the Ad5 genome (Genebank accesion no. M73260), a reporter expression cassette consisting of the constitutive CMV immediate early/gene enhancer and promoter splicing donor and acceptor signals (from pCMVβ, Clontech), *E. coli* ß-galactosidase gene (lacZ), and SV40 poly A, as well as nt 3328 - 5788 of the Ad 5 genome as a homologous recombination fragment. The reporter in this plasmid can be replaced with any gene of interest such as hairpin ribozyme gene for human pCNA or CDC2 kinase mRNA. Recombinant adenoviral vector is generated in 293 cells co-transfected with the pAV shuttle plasmid and pJM17 (Bicrobix Biosystems, Ontario, Canada). The AV plaques on 293 cell monolayer were selected, checked for genomic organization, and plaque-purified for at least two more times as performed routinely (Yei et al., *Human Gene Therapy 5*:731-744, 1994). The resulting adenoviral vector is designated AvCLacZ.

AvCLacZ was propagated in 293 cells and purified as described elsewhere (Yei et al., *Human Gene Therapy* 5:731-744, 1994). The adenoviral vector preparations were then titrated and used for transduction efficacy and toxicity studies. Routinely, titers of recombinant Av vector preparations are between 10^{11} and 10^{12} pfu/ml as measured by plaque assay on 293 cells.

B. Generation of adenoviral vectors that express hairpin ribozyme genes specific for inactivation of human PCNA or cdc2 kinase gene.

Recombinant replication-deficient (E1 deletion) adenoviral vectors (Av) containing ribozyme genes specific for the two targets of restenosis (human PCNA and cdc2 kinase mRNA) were constructed by homologous recombination of shuttle plasmid (pAvCRz; Figure 3) with pJM17 (Bicrobix Biosystems, Ontario, Canada). The shuttle plasmid described above, pAvCLacZ, comprises the following elements: (1) Ad5 sequence 1-452 (Genebank accession no. M73260; containing the left inverted terminal repeat, encapsidation signals and the E1a enhancer), artificial *Xba*I, *Bam*HI and *Xho*I sites, (2) CMV immediate/early gene promoter and enhancer (from pCMVβ expression vector, Clontech; Boshart et al., *Cell 41*:521-530, 1985), artificial *Bam*HI and *Xho*I sites, SV40 splice donor/splice acceptor sequence (from pCMVβ expression vector, Clontech), artificial multiple cloning sites include *Bam*HI, *Not*I, *Bgl*II, *Eco*RV, *Asc*I, *Not*I, *Bam*HI sites in a contiguous arrangement, SV40 polyadenylation (from pCMVβ expression vector, Clontech), artificial *Bam*HI, *Sal*I and *Cla*I sites; and (3) an Ad5 sequence used for homologous recombination (Ad5 sequences 3328-5788). Specific ribozyme genes (see Examples 1 and 2) were cloned into the shuttle plasmid via the BglII and AscI cloning sites.

293 cells were co-transfected with both the shuttle plasmid and pJM17 using $CaPO_4$ or lipofectin method. Recombinant adenoviral vectors formed plaqus and were purified from the transfected 293 monolayers. The resulting AV were further plaque-purified for at least 2 more times as performed routinely (Yei et al., *Human Gene Therapy* 5:731-744, 1994). AV were propagated in 293 cells and CsCl-purified to high titer preparation before evaluating the functions of anti-PCNA or cdc2 kinase Rz.

EXAMPLE 6

IN VITRO CLEAVAGE ASSAYS

Hairpin ribozymes are cloned into *in vitro* transcription vectors (pGEM-7Z, ProMega, Madison, Wis.) and transcribed *in vitro* by T7 RNA polymerase. Following transcription, reactions are treated with DNase and the ribozymes are purified by denaturing polyacrylamide gel electrophoresis. Substrates are then transcribed *in vitro* in the presence [α-^{32}P]UTP and purified by denaturing polyacrylamide gel electrophoresis. The *in vitro* cleavage reactions are carried out by incubating 40 nM ribozyme with 200 nM substrate at 37°C for 0 to 60 minutes in 12 mM $MgCl_2$/2 mM spermidine/40 mM Tris-HCl, pH 7.5. Reactions are terminated by the addition of loading buffer (7 M urea/bromophenol blue/xylene cyanol). Products of the cleavage reactions are resolved by electrophoresis on 15% acrylamide/7 M urea gels and analyzed by autoradiography.

EXAMPLE 7

IN VIVO USE OF RIBOZYMES

A. Experimental Protocol

All animals are treated according to the guidelines of the American Physiological Society. Briefly, a #2 Fr fogarty catheter is used to induce vascular injury in male Sprague-Dawley rats (400 to 500 g in weight). The rats are anesthetized and a cannula is introduced into the left common carotid artery via the external carotid artery. The common carotid artery is then injured by pulling the inflated fogarty catheter through it 3 times. A total of 100 animals are studied and divided into 6 different groups:

Table 9

Group 1	(n=20)	balloon injury alone.
Group 2	(n=15)	balloon injury followed by infusion of saline through an isolated segment.
Group 3	(n=15)	balloon injury followed by local administration of CDC2 kinase ribozyme.
Group 4	(n=12)	balloon injury followed by local delivery of ribozyme to PCNA.
Group 5	(n=25)	balloon injury followed by administration of scrambled sequences of nucleotides resembling CDC2 kinase and PCNA ribozymes.
Group 6	(n+12)	balloon injury followed by local administration of a combination of CDC2 kinase and PCNA ribozymes.

After vessel injury of the common carotid artery, the injured segment is transiently isolated by temporary ligatures. Briefly, two hundred microliters of a combination of lipofectin and hammerhead ribozyme (40 μg) are incubated in the isolated segment for 15 minutes. After the 15 minute incubation, the ligatures are removed. The external carotid artery is ligated and blood flow is restored in the common carotid and the internal carotid artery. The skin wound is then repaired and the animals are transferred to their cages. The animals are then euthanized at 2 weeks and artery is harvested. It s perfusion fixed in formalin and sent for histopathology.

The histopathology sections are then subsequently analyzed by quantitative histology. Using computer facilitated planimetry, the lumen area, area of the intima and area of the media are measured and intimal area to medial area ration is calculated. All values are expressed as mean ± standard deviation and mean ± standard errors of mean. A statistical comparison for each of these parameters is performed between all the groups.

Results of the quantitative histology are shown in Figures 4 and 5 and summarized in Table 10. Briefly, both the cross-sectional area of the intima and the ratio of the intimal area to medial area were significantly reduced in the ribozyme treated arteries compared with those treated with scrambled-sequence polynucleotides or with normal saline. The intimal hyperplasia was inhibited by the CDC-2 kinase ribozyme, the

PCNA ribozyme and their combination. The combination did not seem to have any additive effect.

Table 10

	NO.		INT	I/M
B1	14	MEAN	13.50	0.83
		STDEV	4.47	0.34
B1+NS	8	MEAN	17.74	1.09
		STDEV	6.52	0.42
B1+RZ1	18	MEAN	8.37	0.46
		STDEV	5.04	0.24
B1+SCR	19	MEAN	13.24	0.92
		STDEV	4.43	0.26
B1+RZ2	10	MEAN	7.21	0.43
		STDEV	3.87	0.24
B1+RZoom	10	MEAN	6.218783	0.41197
		STDEV	1.875044	0.141841

B. Additional Assays

1. Tissue Culture Protocols

Smooth muscle cells (SMC) are isolated from rat aorta and maintained in DMEM medium and 10% FBS. MTT assay: This is a quantitative colorimetric assay for cell proliferation and survival. Rat SMC's (passage 4-8) are seeded into 96 well plate (1500 cells/well) one day before treatment. Cells are then treated with 2mM of CDC-2 kinase/PCNA ribozyme and 4mM lipofectin for 1 hour. A second dose of ribozyme (4mM) is added on day 2. On day 3, 10mL of MTT is added into each well

for 4 hours. The dye in the cells is extracted in DMSO after washing off any supernatant dye from the well. The OD is measured with microplate reader at 590 mM.

The MTT assay using PCNA ribozyme demonstrates significant inhibition of cell proliferation in cell culture as measured by uptake of MTT in comparison to scrambled sequence treated cells and control cells.

2. Quantification of mRNA

SMC's (4-8 passage) are seeded into culture dish one day prior to treatment. RNA is extracted from the cells after treatment with ribozyme, scrambled sequence polynucleotide, 10% FBS or serum free medium for 2 or 6 hours. RT-PCR is then performed utilizing RNA-PCR kit from Perkin Elmer. An appropriated primer sequence for CDC-2 kinase or PCNA is used for analysis. A beta-actin primer is used to ensure that the amount of RNA loaded in each well is approximately equal.

RT-PCR studies using CDC-2 kinase ribozyme show reduction in the CDC-2 kinase mRNA at 2 hours and further reduction at 6 hours in comparison to controls. To ensure that equivalent amount of RNA is loaded in each well, RT-PCR is performed using a primer for beta-actin which shows similar levels of beta-actin mRNA in each group.

3. Protein Expression

Three types of protein assays may also be accomplished, including a) Western blotting; b) Biosynthetic labeling with 35S labeled methionine followed by immunoprecipitation of radiolabelled protein as a measure of newly synthesized target protein; and c) Histone H1 kinase assay for CDC-2 kinase. The Histone H1 kinase assay is a functional assay for CDC-2 kinase and measures the amount of p32 labeled phosphate transferred from ATP to Histone H1.

4. Anti-PCNA and CDC2 Western Blotting

Cells were lysed by boiling in 1 x SDS sample buffer. Protein concentration of the cell lysate was determined by Bradford assay (USB). Next, 25 ug of total protein was loaded onto 14% Tris-glycine gel (Novex) and separated from each

other by electrophoresis. The protein gel was then semi-dry transferred to PVDF Immobilon membrane (Millipore). Amido black stain and photograph were performed to verify equal protein loading. The membranes were blocked with 5% milk and incubated with specific primary antibody (anti-rat PCNA monoclonal antibody, Immunotech, or anti-human cdc-2 monoclonal antibody, Pharmingen) at 1 ug/ml in 5% milk/ 1XPBS for 4 hours room temperature. The blots were washed thoroughly with 1x PBS/ 0.05% Tween-20 and incubated with HRP-conjugated goat anti-mouse IgG (Chemicon) for 1 hour. The blots were then developed via ECL (Amersham) and exposed to film. PCNA produced a 36 kD band, whereas cdc2 kinase produced a 34 kD band.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Immusol, Incorporated

(ii) TITLE OF INVENTION: RIBOZYME THERAPY FOR THE TREATMENT AND/OR PREVENTION OF RESTENOSIS

(iii) NUMBER OF SEQUENCES: 35

(iv) CORRESPONDENCE ADDRESS:
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(C) CITY: Seattle
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(F) ZIP: 98104-7092

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE: 12-SEP-1996
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: McMasters, David D.
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(ix) TELECOMMUNICATION INFORMATION:
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(B) TELEFAX: (206) 682-6031

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

NNNBNGUCNN NNNN 14

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

NNNBNGUCNN NNNNN 15

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

NNNBNGUCNN NNNNNN 16

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

NNNBNGUCNN NNNNNNN 17

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

NNNBNGUCNN NNNNNNNN 18

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AGTCAGTCTT CAGGAT 16

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TCCTGGTCAG TACATG 16

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CTGGGGTCAG CTCGTT 16

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TCCGAGTCAC CAGGAA 16

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CCAGTGTCTG AGCCAG 16

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CCTGTGTCAG GCTTTC 16

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AAGCAGTCAG ACCAAA 16

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ACTGGGTCGG GAAGTC 16

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TGACTGTCTC CATTAT 16

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GCCTGGTCCA GGGCTC 16

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GACTCGTCCC ACGTCT 16

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CTGCGGTCTG AGGGCT 16

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TTTCTGTCAC CAAATT 16

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CCGCCGTCCC TGGTGC 16

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CTGGAGTCAC CGCTGG 16

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CGCCCGTCAC TGGTTC 16

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GTACGGTCTC CCAGAC 16

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CAGGCGTCCA CGTACG 16

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

AAACTGTCTG GCCAGT 16

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

TTTCTGTCTT GAAGAC 16

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

TACAGGTCAA GTGGTA 16

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

AAATTTCTCT ATTAAAG 17

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

AACGAGCTAG AACCAGACCA GAGAAACACA CGTTGTGGTA TATTACCTGG TA 52

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CTGGCTCAAG AACTGGACCA GAGAAACACA CGTTGTGGTA TATTACCTGG TA 52

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CCAGCGGTAG AACCAGACCA GAGAAACACA CGTTGTGGTA TATTACCTGG TA 52

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

AGCCCTCAAG AAGCAGACCA GAGAAACACA CGTTGTGGTA TATTACCTGG TA 52

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

AACGAGCTCT GATGACCTCG TGAGAGGGAA ACCCCAG 37

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

CTGGCTCACT GATGACCTCG TGAGAGGGAA ACACTGG 37

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

CCAGCGGTCT GATGACCTCG TGAGAGGGAA ACTCCAG 37

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

AGCCCTCACT GATGACCTCG TGAGAGGGAA ACCGCAG 37

Claims

We claim:

1. A hairpin or hammerhead ribozyme having the ability to inhibit abnormal smooth muscle cell proliferation in vascular tissue.

2. The ribozyme of claim 1, wherein the ribozyme has the ability to inhibit the activity of a growth factor responsible for abnormal smooth muscle cell proliferation in vascular tissue.

3. The ribozyme of claim 1, wherein the ribozyme inhibits the activity of cdc2-kinase, PCNA, Cyclin B1 or Lysyl Oxidase.

4. The ribozyme of claim 2, wherein the ribozyme specifically cleaves a protein responsible for the production of an extracellular matrix protein.

5. The ribozyme of claim 1, wherein the ribozyme has the ability to prevent abnormal deposition of extracellular matrix in vascular tissue.

6. A nucleic acid molecule encoding the ribozyme of claim 1.

7. The nucleic acid molecule of claim 6, wherein the nucleic acid is DNA or cDNA.

8. The nucleic acid molecule of claim 6, under the control of a promoter to transcribe the nucleic acid.

9. A host cell comprising the ribozyme of claim 1.

10. A vector comprising the nucleic acid of claim 6.

11. A host cell comprising the vector of claim 10.

12. The vector of claim 10, wherein the vector is a plasmid, a virus, retrotransposon or a cosmid.

13. The vector of claim 12, wherein the vector is an adenoviral vector or an AAV.

14. A host cell stably transformed with the vector of claim 13.

15. The host cell of claim 9 or 14, wherein the host cell is a human cell.

16. A method for producing a ribozyme, the ribozyme being able to inhibit abnormal smooth muscle cell proliferation in vascular tissue, comprising providing DNA encoding the ribozyme under the transcriptional control of a promoter, transcribing the DNA to produce the ribozyme.

17. The method of claim 16, wherein the ribozyme is produced *in vitro*.

18. The method of claim 17, further comprising purifying the ribozyme produced.

19. The method of claim 16, wherein the ribozyme is produced *in vivo*.

20. A method of inhibiting abnormal smooth muscle cell proliferation in vascular tissue, comprising introducing into a cell an effective amount of the ribozyme of claim 1.

21. A method of inhibiting abnormal smooth muscle cell proliferation in vascular tissue, which comprises introducing into the cell an effective amount of the DNA of claim 6 under conditions favoring transcription of the DNA to produce the ribozyme.

22. The method of claim 20 or 21, wherein the cell is a human cell.

23. A method of preventing abnormal smooth muscle cell proliferation in vascular tissue, which comprises introducing into the cell an effective amount of the DNA of claim 6 under conditions favoring transcription of the DNA to produce the ribozyme.

24. The method of claim 23, wherein the cell is a human cell.

25. A method of inhibiting or preventing abnormal smooth muscle cell proliferation in vascular tissue in a subject, which comprises administering to the subject an effective amount of the ribozyme of claim 1.

26. The method of claim 25, wherein the ribozyme is delivered to the smooth muscle cell exoluminally, transluminally, by stent, by a biodegradable polymer or sphere or in a pleuronic gel.

FIGURE 1

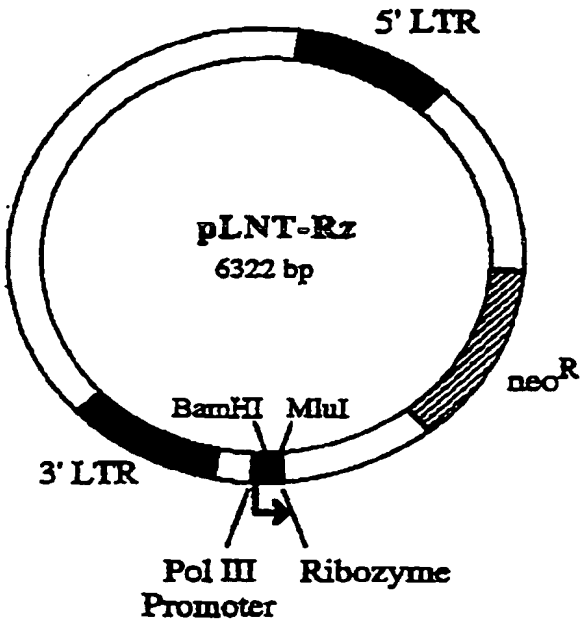

5' LTR
pLNT-Rz
6322 bp
neo^R
BamHI
MluI
3' LTR
Pol III
Promoter
Ribozyme

FIGURE 2

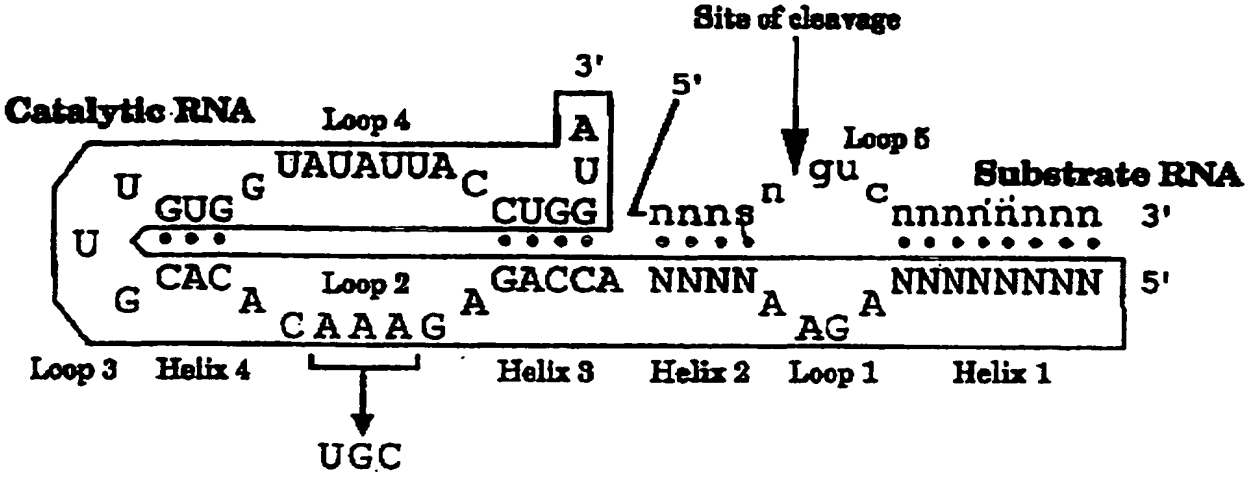

Site of cleavage
3'
5'
Catalytic RNA
Loop 4
Loop 5
Substrate RNA
UAUAUUA
GUG
CUGG
nnns
gu
nnnnnnnn
3'
CAC
Loop 2
GACCA
NNNN
NNNNNNNN
5'
CAAAG
AG
Loop 3
Helix 4
Helix 3
Helix 2
Loop 1
Helix 1
UGC

FIGURE 3

pAvCRz

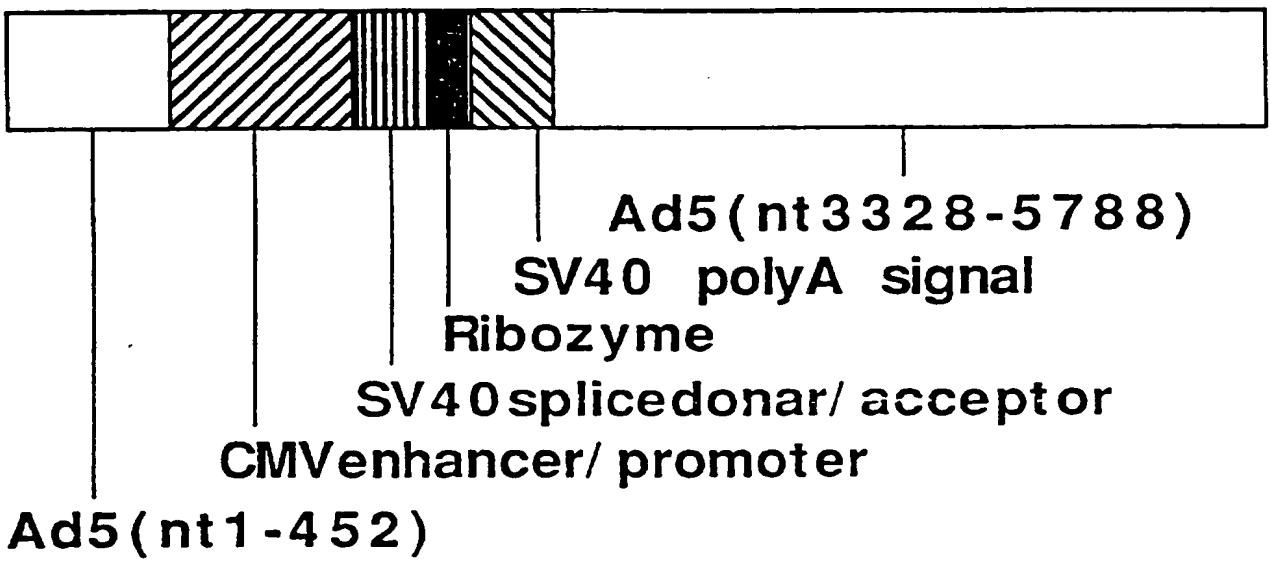


FIGURE 4

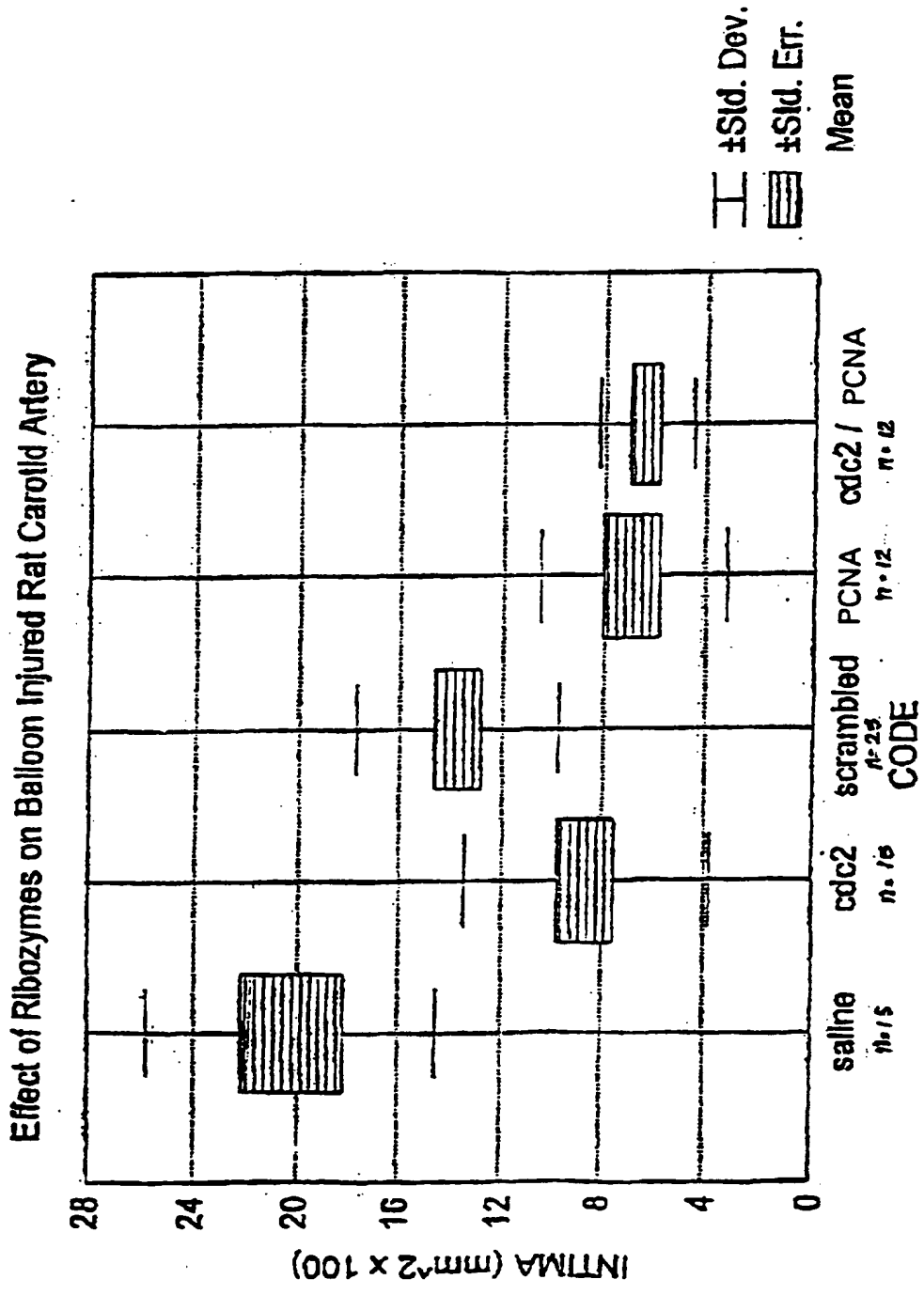

Effect of Ribozymes on Balloon Injured Rat Carotid Artery
INTIMA (mm^2 x 100)
28
24
20
16
12
8
4
0
saline
n=15
cdc2
n=16
scrambled
n=25
PCNA
n=12
cdc2 / PCNA
n=12
CODE
±Std. Dev.
±Std. Err.
Mean

FIGURE 5

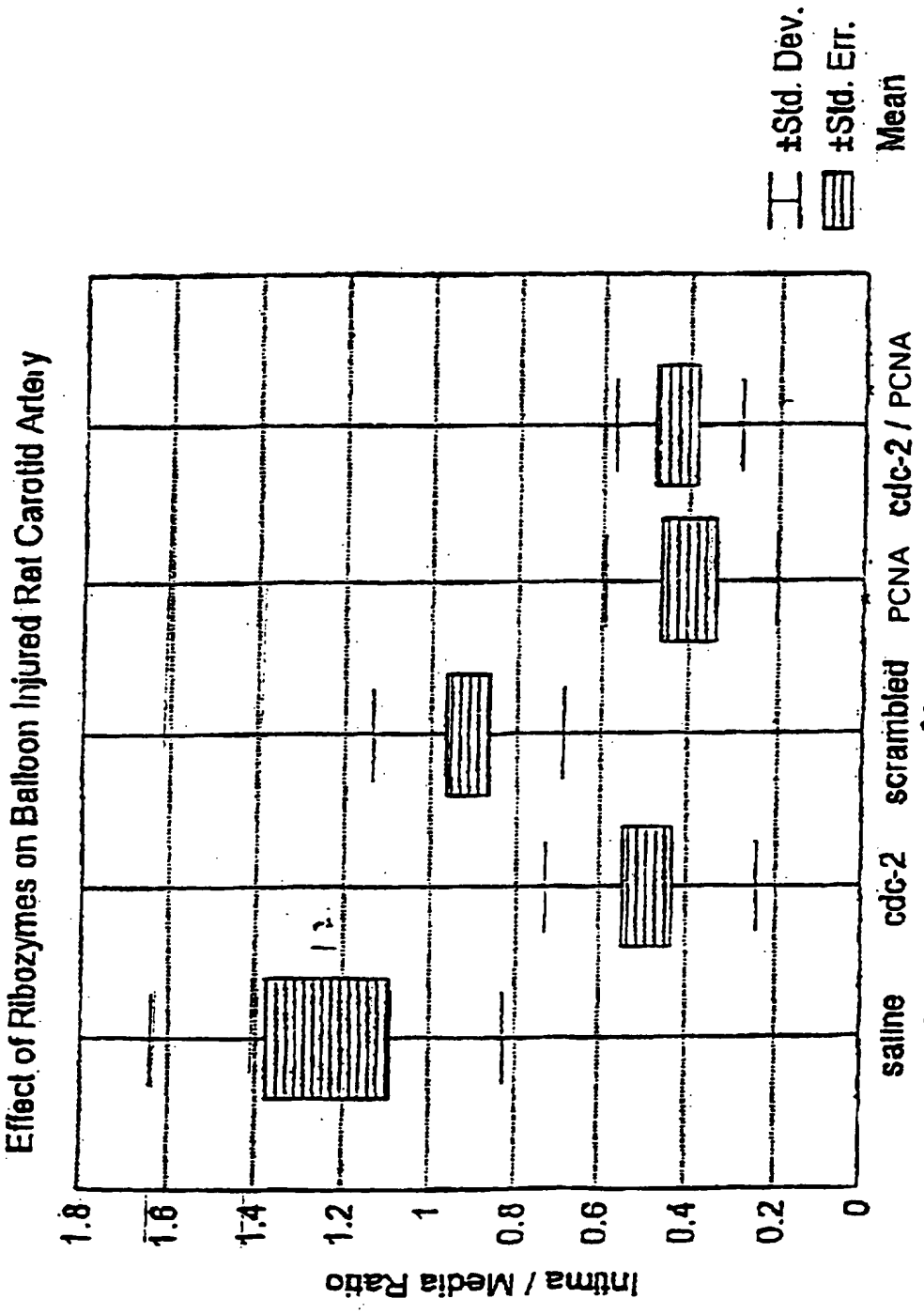

Effect of Ribozymes on Balloon Injured Rat Carotid Artery
Intima / Media Ratio
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
saline
cdc-2
scrambled
PCNA
cdc-2 / PCNA
n = 15
n = 15
n = 25
n = 12
n = 12
±Std. Dev.
±Std. Err.
Mean

FIGURE 6

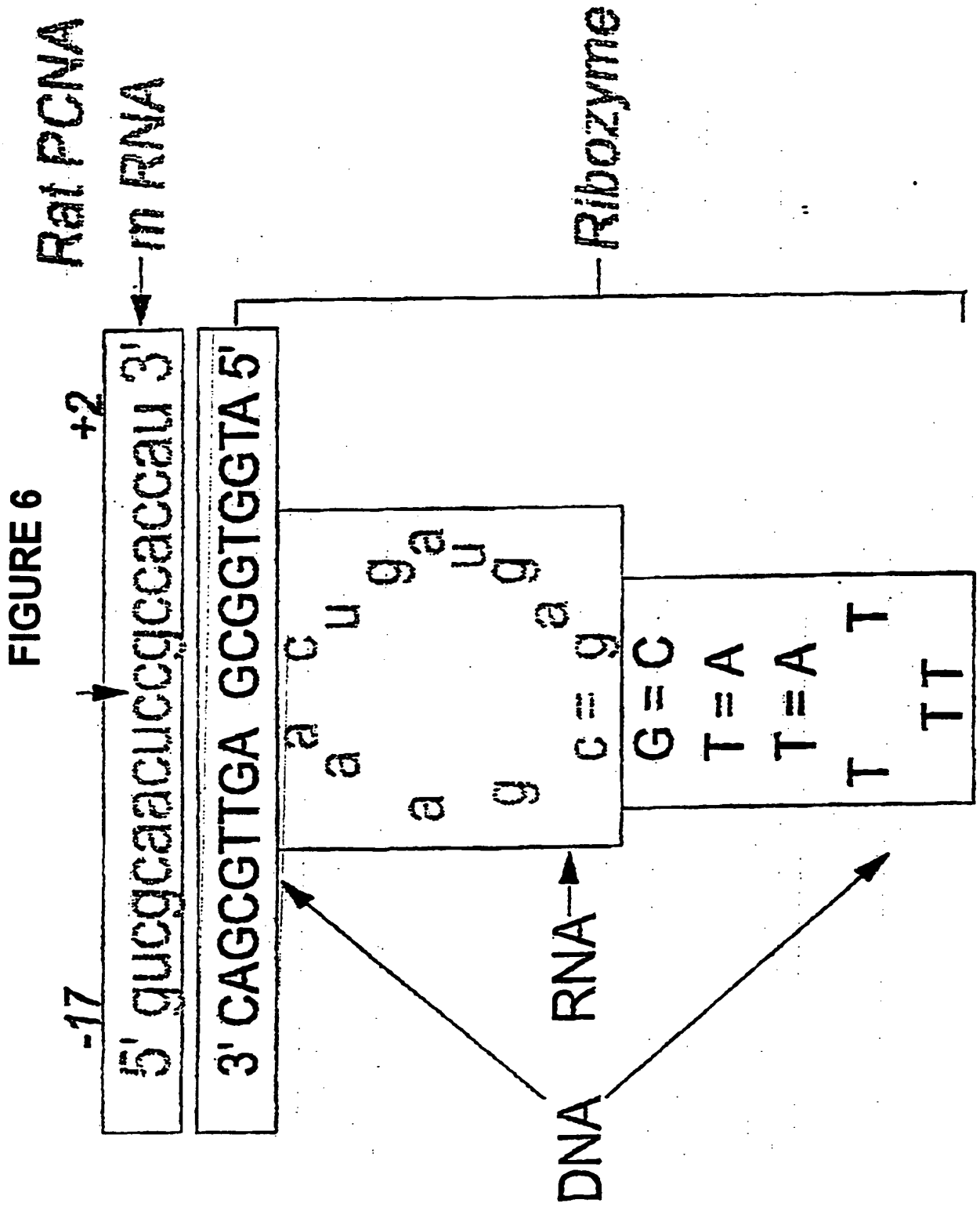
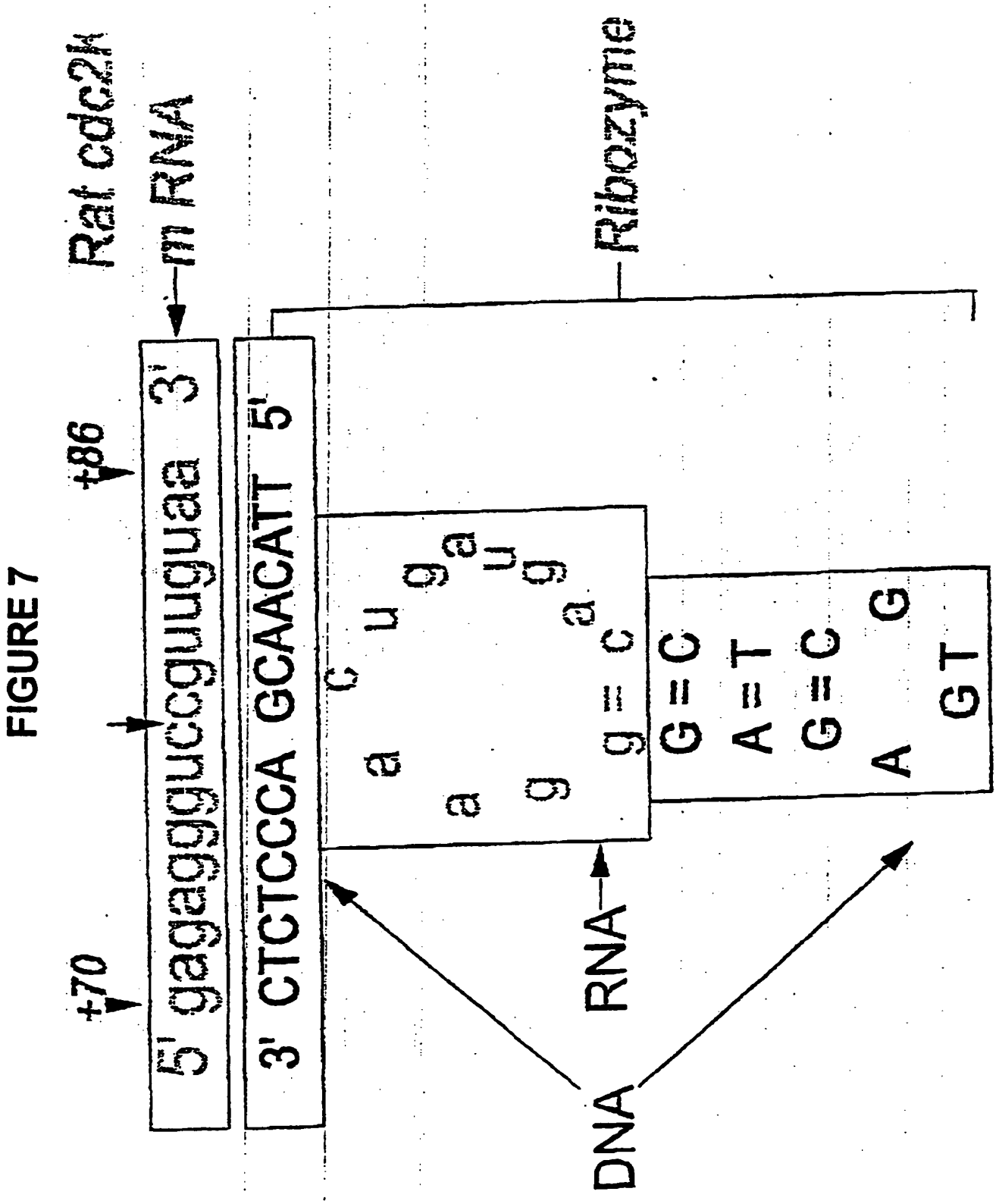

Rat PCNA
m RNA
Ribozyme
+2
-17
5' gucgcaacuccgcaccaau 3'
3' CAGCGTTGA GCGGTGGTA 5'
a c u g a u g
a a g c = g a
G = C
T = A
T = A
T T
T T
DNA
RNA

FIGURE 7


Rat cdc2K
m RNA
Ribozyme
+86
+70
5' gagaggcguuguaa 3'
3' CTCTCCCA GCAACATT 5'
c u g a u g
a g g = c a
G = C
A = T
G = C
A G
G T
RNA
DNA